AD_____

Award Number: W81XWH-12-2-0011

TITLE: Optimization and Implementation of Long Nerve Allografts

PRINCIPAL INVESTIGATOR: David F. Muir

CONTRACTING ORGANIZATION: University of Florida
Gainesville, FL 32611-5500

REPORT DATE: March 2013

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
March 2013	Annual	01 February 2012 - 31 January 2013

4. TITLE AND SUBTITLE

Optimization and Implementation of Long Nerve Allografts

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-12-2-0011

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
David F. Muir

E-Mail: muir@mbi.ufl.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Florida
Gainesville, FL 32611-5500

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
Loss of nerve function occurs when a nerve is severed. Any significant loss of nerve tissue requires a graft to restore continuity and promote nerve regeneration and recovery of function. Presently, there is no acceptable nerve grafting method for the repair of extensive nerve damage. An acellular nerve allograft product is available for the reconstruction of short to moderate length nerve defects, however, in its present from the allograft is not suitable for the repair of lengthy nerve defects. The aims of this project are to evaluate several long (7cm) nerve allograft technologies. All tasks scheduled for year 1 of this two-year project were completed. A nerve graft processing protocol was established for rabbit peripheral nerve that mimics the production of the human nerve allograft product used clinically. This includes detergent decellularization, treatment with chondroitinase ABC, and irradiation sterilization. A 7-cm, stepping-stone graft was established. A tubular nerve protector biologic was devised. A long nerve allograft model in rabbit was established, including several neurological evaluation methods. Control conditions include a standard acellular allograft and cellular autograft (the gold standard in nerve grafting). At this point, all nerve grafting has been completed on schedule and evaluations of nerve regeneration and recovery of function have begun. If successful this project will establish technologies for the repair of extensive nerve damage using off-the-shelf nerve products that overcome the limitations of nerve autografting.

15. SUBJECT TERMS
peripheral nerve, nerve graft, allograft, nerve regeneration, rehabilitation

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	8	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

INTRODUCTION

Loss of nerve function is complete when a nerve is severed and significant recovery without surgical intervention is rare. Any significant loss of nerve tissue requires a graft to restore continuity and promote nerve regeneration. Presently, there is no acceptable nerve grafting method for the repair of extensive nerve damage. Recently, an acellular nerve allograft product was brought to market that is now used widely for the reconstruction of short to moderate length nerve defects. However, this allograft, in its present from, is not suitable for the repair of lengthy nerve defects, like those often encountered with battlefield trauma. This proposal aims to greatly expand the application of nerve allografts for the reconstruction of extensive nerve damage. One approach to apply an acellular allograft in the repair of long nerve defects is the stepping-stone graft. Stepping-stone grafts combine two moderate length allografts with a small, interposed autologous nerve segment. A second approach is to apply a biodegradable gel coating to protect and prolong the efficacy of the acellular graft once implanted.

Specific Aims are to: 1) Evaluate 7 cm acellular allograft prototypes 4 weeks after implantation in rabbits. Key aspects of graft viability and integration will be assessed to finalize grafting protocols. 2) Conduct a 26-week preclinical evaluation of 7 cm stepping-stone allografts (SS-grafts) and 7 cm hydrogel-coated allografts in the reconstruction and rehabilitation of extensive peripheral nerve injury in rabbits. Control conditions include a standard acellular allograft and cellular autograft (the gold standard in nerve grafting). Outcomes will be evaluated by translational neurological testing.

BODY

The Award period began official on February 1, 2012. However, administration to release funds for this project was completed March 6, 2012. Despite this delay, excellent progress was made and our work is on schedule.

The early Milestone to obtain ACURO approval for animal use was accomplished.

This project requires several key personnel including the new hire of a full-time research associate (stated as To-be-Announced in the Budget). We were successful in recruiting a highly qualified, post-doctoral associate, Anne Engemann, Ph.D. Due to the aforementioned administrative delays, Dr. Engemann started in this position as of April 2, 2012.

Task 1. Evaluate nerve grafts in 4-week study (months 1-6):

Task 1a. Collect, process (decellularize) and prepare 7 cm acellular allografts (n=16)(8 rabbits)(months 1-3).

i) Evaluate detergent decellularization, including extraction of chondroitin sulfate chains (CS56 immunolabeling), myelin (sudan black staining), axons (neurofilament immunolabeling), and DNA (propidium iodide staining).

ii) Evaluate integrity of decellularized grafts, including retention of nerve sheaths (laminin immunolabeling) and CSPG core proteins (CSPG neo-epitope immunolabeling).

iii) Test methods for thorough coating of grafts with hydrogel (spiked with dye).

Progress:

Task 1a was completed successfully. Nerves were collected from donor rabbits and stored frozen. Test samples were processed by several detergent decellularization schemes and then evaluated by numerous histological methods to assess cellular extraction and retention of the nerve sheaths.

The goal was to establish a decellularization method for rabbit nerve that is equivalent to the Avance® processing used by AxoGen Inc for their human nerve allograft product. As this processing method is proprietary we are unable to know the exact Avance® protocol. We do, however, know the published methods on which the Avance® protocol is based. (Also, we have direct insights to this process because it combines technologies developed in my lab and that of C. Schmidt at UT, Austin.) In addition, AxoGen was kind enough to perform the Avance® process on a set of rabbit peroneal nerves for comparison in our study. Nerves processed by the exact Avance® method are referred to as DC1. We tested two methods for detergent decellularization. One is as close to the Avance® method as we could ascertain, referred to as DC2. The other method involved a modification by eliminating one detergent step, referred to as DC3. All three processing methods included the same final treatment step with chondroitinase ABC. We evaluated the outcome of the three processing methods by several histological techniques. These included hematoxylin and eosin, sudan black, Hoescht, laminin, neurofilament, S-100, CS56 and CS-6. Results of each method were semi-quantitatively scored (0=Not extracted, 1=Partially extracted, 2=Mostly extracted, 3=Fully extracted and R= Redistributed).

Results for three detergent processing schemes (DC1, DC2, DC3) are shown in Figures 1-8. Based on these evaluations a final allograft processing protocol was established. The DC2 protocol rendered rabbit nerve similar to the nerves processed by the Avance® method (DC1). Rabbit peroneal nerve allografts (n=18, including 2 backups) were processed by protocol DC2. Nerve trims were evalauted as describe above and all criteria for nerve graft processing were met.

Next, various hydrogel types, concentrations and application methods were tested on nerve segments in vitro. The hydrogel product DuraSeal was most effective using the concentration and instructions provided by the manufacturer for use in other tissues. A trough (mold) was developed to aid application and retention of the hydrogel around the nerve until it polymerized. An optimal volume of hydrogel per cm of nerve was established.

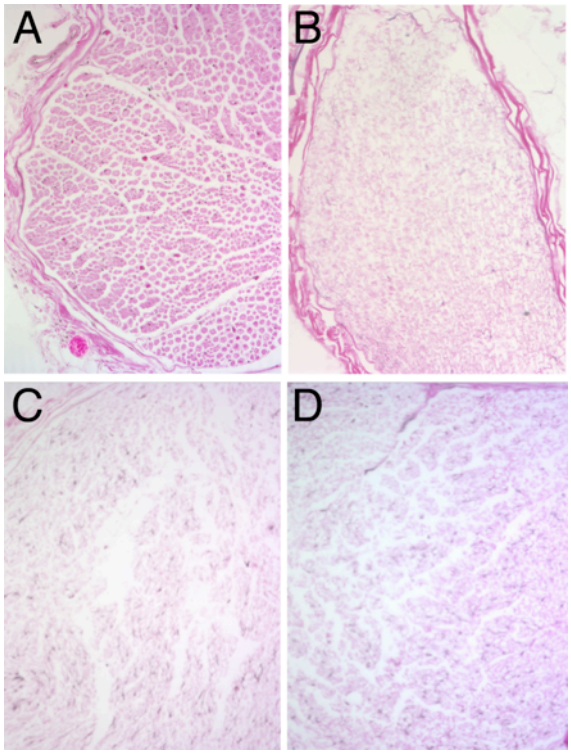

Figure 1. H & E Staining.

Panel	Score
A. Normal nerve control	0
B. DC1	2
C. DC2	2
D. DC3	2

H&E staining for general histology showed the three processing methods achieved extensive cellular extraction and retention of overall nerve sheath integrity.

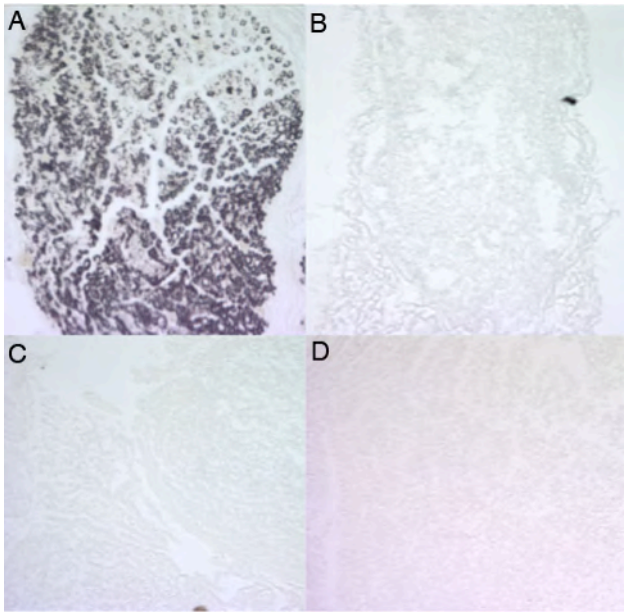

Figure 2. Sudan Black Staining.

Panel	Score
A. Normal nerve control	0
B. DC1	3
C. DC2	3
D. DC3	3

Sudan black staining for myelin showed the three processing methods to be equally and highly effective at extracting myelin.

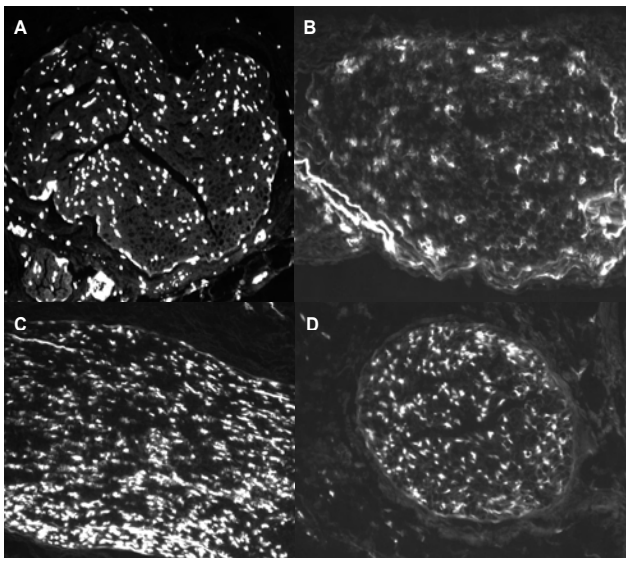

Figure 3. Hoescht Staining.

Panel	Score
A. Normal nerve control	0
B. DC1	2R
C. DC2	1R
D. DC3	1R

Hoescht staining for DNA showed the three processing methods did not eliminate DNA but rather dispersed and redistributed chromosomes.

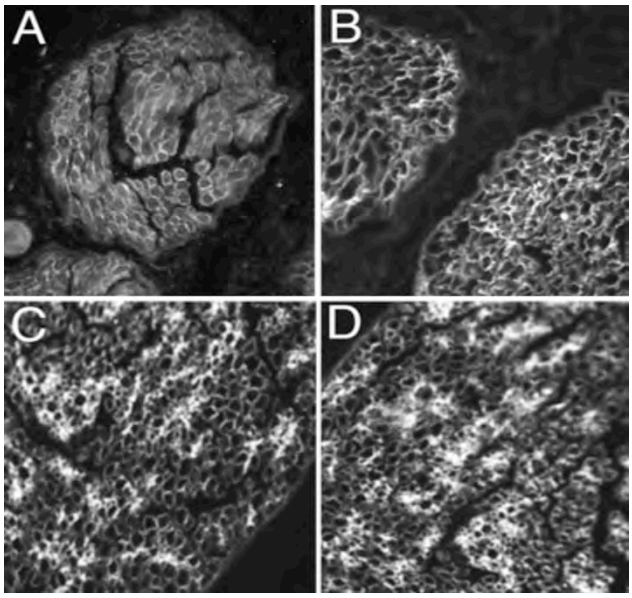

Figure 4. Laminin Immunostaining.

Panel	Score
A. Normal nerve control	0
B. DC1	2R
C. DC2	1R
D. DC3	1R

Laminin is a major component of the nerve sheaths including the important basal lamina surrounding axons. Laminin immunolabeling showed the three processing methods had an equal effect on the preservation of basal lamina structure.

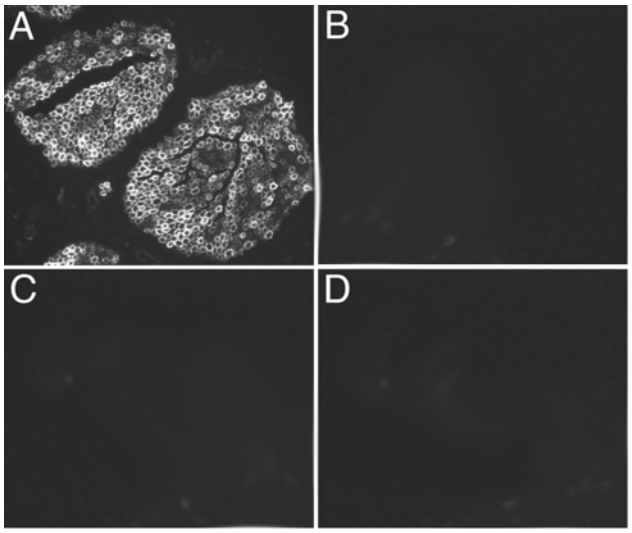

Figure 5. S-100 Immunostaining.

Panel	Score
A. Normal nerve control	0
B. DC1	3
C. DC2	3
D. DC3	3

S-100 immunolabeling (a cytoplasmic protein in Schwann cells) showed the three processing methods equally and very effectively extracted the cytoplasm of Schwann cells.

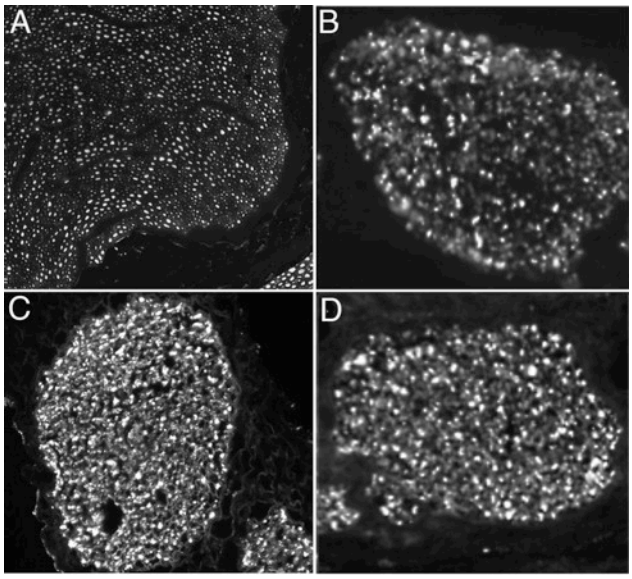

Figure 6. NAP-4 Neurofilament Staining.

Panel	Score
A. Normal nerve control	0
B. DC1	1R
C. DC2	1R
D. DC3	1R

Neurofilament immunolabeling (a marker of the neuronal/axonal cytoskeleton) showed the three processing methods only partially extracted and redistributed the axonal cytoskeleton.

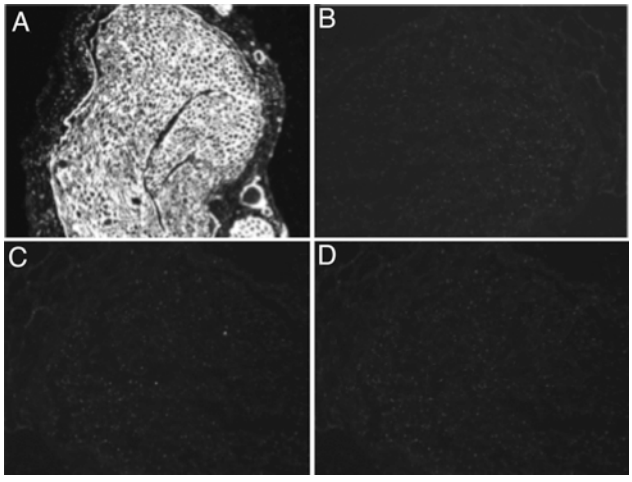

Figure 7. CS56 Immunostaining.

Panel	Score
A. Normal nerve control	0
B. DC1	3
C. DC2	3
D. DC3	3

CS56 antibody labels the side-chains of chondroitin sulfate proteoglycans. All processing methods include the same treatment step with chondroitinase ABC, an enzyme that degrades the side-chains. CS56 immunolabeling showed the three processing methods effectively eliminate the chondroitin sulfate side-chains after detergent extractions.

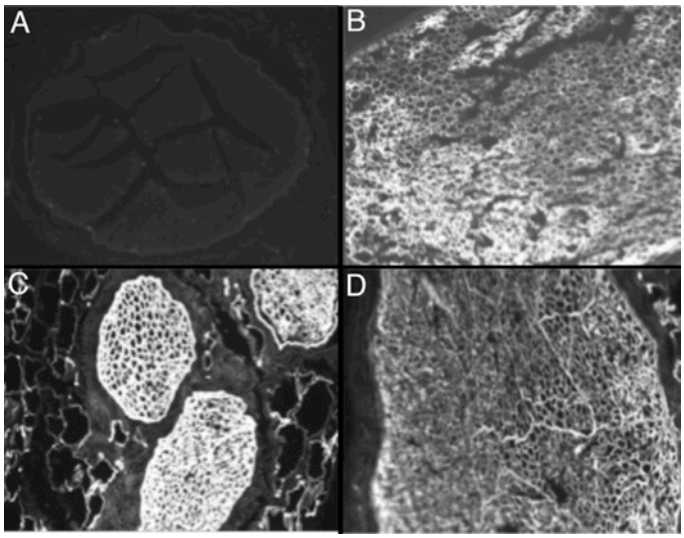

Figure 8. CS-6 Neoepitope Immunostaining.

Panel	Score
A. Normal nerve control	0
B. DC1	3
C. DC2	3
D. DC3	3

CS-6 neoepitope immunolabeling reveals proteoglycan core protein that was stripped of CS-6 side-chains by chondroitinase ABC treatment. Positive C6-6 immunolabeling showed the three processing methods effectively eliminate the chondroitin sulfate side-chains and yet the core protein remains intact in the basal lamina.

Task 1b. Implant nerve allografts in rabbits (16 rabbits) (months 2-4).
i) Perform nerve grafting with control (undivided, uncoated) DCI-grafts (n=4).
ii) Perform nerve grafting with DCI-grafts coated with hydrogel (n=4).
iii) Perform nerve grafting with SS-grafts in the 3-1-3 configuration (n=4).
iv) Perform nerve grafting with SS-grafts in the 4-1-2 configuration (n=4).

Progress
Implantation of the four nerve allograft types was completed on schedule and without complications. All animal hosts survived and remained in good health throughout the 4-week survival period.

Task 1c. Evaluate outcomes of nerve grafting after 4 weeks, including:
i) Axon morphometrics (scoring neurofilament immunopositive axons)
ii) Graft integrity, cellularity and viability within the graft (H&E staining)
iii) Schwann cell migration from autologous nerve segment (S100 immunolabeling).

Progress
Task 1C was completed and the goals of the 1-month grafting study were achieved. All graft hosts were terminated and the engrafted nerves removed. Nerves were processed, sectioned and histology and immunocytochemistry was performed. Axon morphometrics indicated nerve regeneration occurred and advanced well into all of the graft types. As expected, because the proximal aspect of all grafts was the same, no significant differences were found in initial nerve regeneration or proximal graft integrity (see Fig. 9).

Differences in the distal grafts (not yet occupied by regenerating nerve) were readily observed, as expected. Based on our observations the following conclusions were made:

1) DCI-grafts (control) were integrated into the recipient nerve and appeared to be immunotolerated well. As expected, the onset of vacuolization and deterioration was observed in the distal aspect of the grafts.

2) The outcome of DCI-grafts coated with hydrogel was complex and unclear. The hydrogel evoked an unusual inflammatory response. Furthermore, there was considerable variation in the hydrogel coating that in some instances failed to remain intact in vivo. These findings indicated the hydrogel coating condition was problematic, required further development and, therefore, was unsuitable at this point for our long-term grafting study. In an earlier project we performed

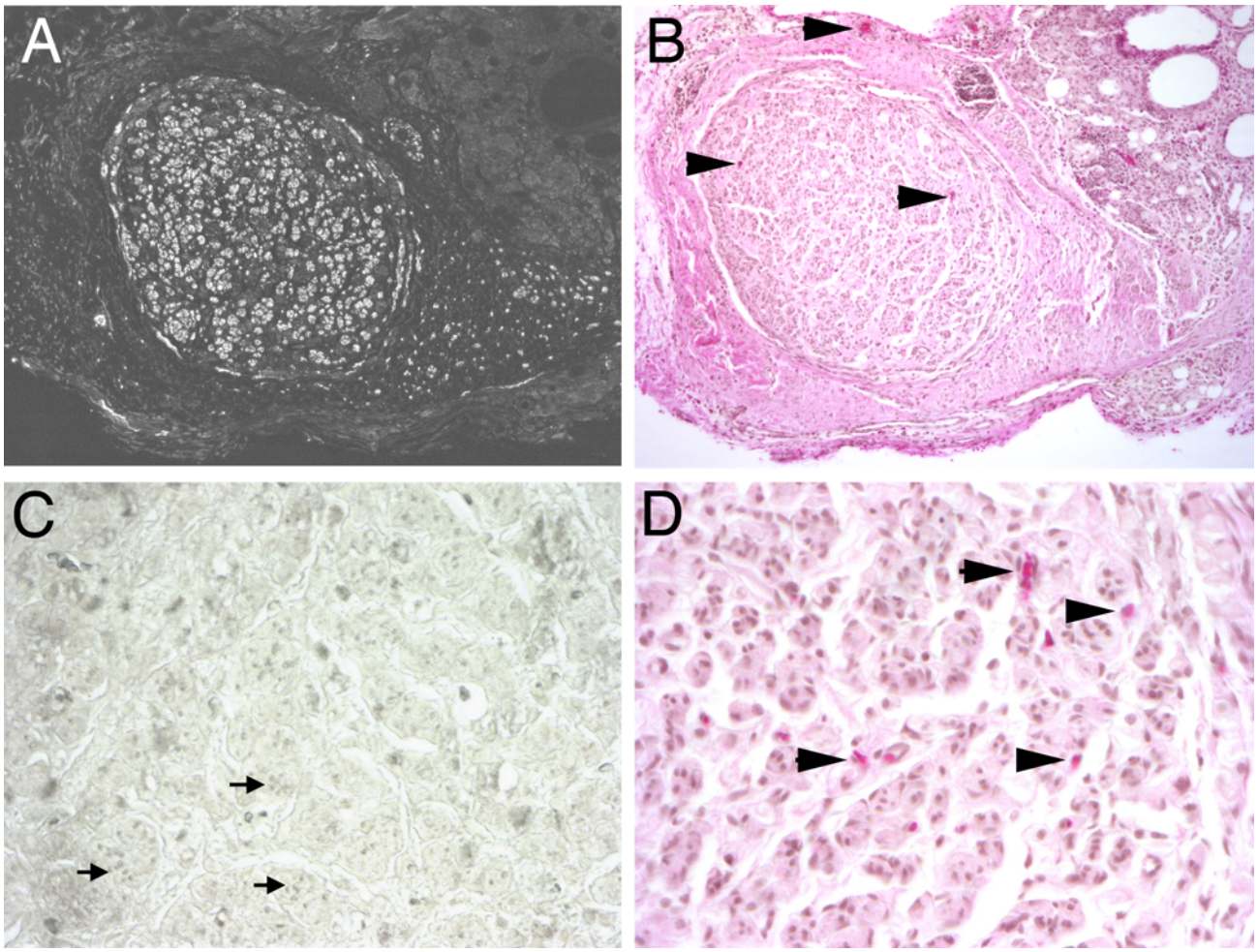

Figure 9. Representative photomicrographs of the proximal DCI-graft component, 4 weeks post-implantation.

A: Neurofilament immunolabeling for axons showed extensive nerve regeneration into the grafts.
B: H&E staining indicated grafts were integrated and immunotolerated. (Arrowheads: blood vessels)
C: Sudan black stained clusters of myelinated axons (arrows) indicating nerve maturation within the graft.
D: H&E staining (high mag of B) showed extensive blood filled capillary network within the grafts (arrowheads).

preliminary testing using a very different approach to protect the DCI-grafts in vivo. This involved placing the distal half of the graft inside a biodegradable, natural biologic tube. This tube is a multi-laminar extracellular matrix from small intestine submucosa (SIS), marketed as the AxoGuard® Nerve Protector (AxoGen Corp). This FDA-approved material is used in numerous clinical applications. Applied to nerve, the SIS tube initially prevents the influx of inflammatory cells and protects the nerve graft from degradation. Importantly, the SIS tube is eventually remodeled to an epineurial sheath-like structure. The time course of protection and remodeling seems ideally suited for use with long nerve grafts. Also, the tubes are preformed, eliminating the variable encountered with application of the hydrogel. The tube will be placed around the distal half of the nerve graft to protect it for several months, while allowing normal early cell repopulation and nerve regeneration within the proximal part of the nerve graft (not protected by the tube). This modification to our animal use protocol was approved by our IACUC and then by ACURO.

3) All SS-grafts well immunotolerated well. One SS-graft lack continuity with the proximal recipient nerve (the sutures did not hold) and was excluded from the evaluation. SS-grafts contained viable Schwann cells migrating from the live autograft segment into the flanking DCI graft segments,

as hoped. The SS-grafts with a 4-1-2 configuration (compared to the 3-1-3) showed more extensive Schwann cell population, especially toward the distal end of the graft. This outcome is optimal and the 4-1-2 configuration was selected for further testing in long-term grafting.

Based on these outcomes, graft constructs and configurations for long-term study were established (see Task 2b).

Task 2. Evaluate nerve grafts in 26-week study (months 7-24):
Task 2a. Collect, process and prepare 7 cm acellular allografts (18 rabbits)(months 7-10).

Progress

Thirty-eight peroneal nerves were collected from 19 donor rabbits. The grafts were decellularized and processed by the protocol established in Task 1. The grafts were then frozen, packaged and shipped to a contract facility for gamma irradiation sterilization. After irradiation, the return shipment was severely damaged in transport by a commercial service. The grafts had thawed and the sterile packaging was breached rendering the grafts usable for in vivo grafting. A modification to the IACUC protocol was submitted to allow for replacement donor animals. The protocol modification was approved by our IACUC and then by ACURO. Replacement rabbits were obtained. The nerves were harvested and processed successfully. Test samples met the histological criteria established in Task 1 and passed in vitro sterility tests. Despite having to repeat the collection and production of nerve grafts we remained on schedule for long-term grafting experiments. This Task was completed successfully.

Task 2b. Implant nerve grafts in rabbits (48 rabbits) (months 8-14).

Progress

The implantation of the four nerve graft types was completed ahead of schedule. As stated above, the hydrogel coated nerve graft condition was replaced with the SIS tube encased nerve graft. Also, two SS-graft configurations were evaluated in Task. The SS-grafts with 4-1-2 configuration had more extensive Schwann cell population, especially toward the distal end of the graft. This outcome is optimal and the 4-1-2 configuration was selected for further testing in long-term grafting. The revised design is shown in Table 1.

Table 1. 26-week evaluation of nerve graft technologies (revised)

Nerve graft (7 cm)	Sample	End-point	Evaluations
Autograft	n=12	26 weeks	• Toe-spread function • Trans-graft electrophysiology • Axon morphometrics • Graft integrity, cellularity, viability
DCI-graft	n=12		
DCI-graft + SIS tube	n=12		
SS-graft (4-1-2)	n=12		

All nerve grafting surgeries were performed successfully. All graft recipients tolerated the surgery without complications and remain in good health. As anticipated, some recipients have a propensity to chew their foot due to the imposed loss of sensory nerve function. This transient condition is monitored closely and managed proactively by the application of gauze wraps and bitter-tasting spray. Thus far, no detrimental self-mutilation has occurred. Animals are monitored daily and health concerns addressed. To date, no animals have met terminated criteria or have been eliminated from the protocol. Function testing (Task 2c) is underway.

Future Work

Task 2c. Assess progressive recovery of function after nerve repair (months 12-20).

Task 2d. Perform trans-graft electrophysiology 26 weeks after grafting (months 14-20).

Task 2e. Remove grafts; perform histological/immunocytochemical examination (months 14-22).

Task 2f. Perform histomorphometric scoring for nerve regeneration (months 18-24).

Task 2g. Analyze and document results (months 20-24).

KEY RESEARCH ACCOMPLISHMENTS:

- A nerve graft processing protocol was established for rabbit peripheral nerve that mimics the production of the human nerve allograft product (Avance®) used clinically. This includes detergent decellularization, treatment with chondroitinase ABC , and irradiation sterilization.
- A long stepping-stone graft (with a live autograft segment interposed between two DCI-graft segments) was established.
- A nerve allograft model in rabbit was established to test graft variables and outcomes for the repair of extensive nerve damage using long nerve grafts.
- Test nerve grafts were implanted successfully and on schedule.

CONCLUSION: The Tasks completed in year 1 established a novel decellularized nerve allograft model in rabbit. Several nerve allograft prototypes were examined and protocols established for long-term in vivo evaluation. The goal is to discover ways to increase the effective length of decellularized nerve allografts. Presently nerve allografting is limited to the repair of moderate (1-4 cm) nerve gaps. This project will test four grafting technologies involving 7 cm nerve allografts in a small diameter rabbit nerve model. At this point, all nerve grafting has been completed on schedule and evaluations of nerve regeneration and recovery of function have begun. If successful this project will establish technologies for the repair of extensive nerve damage using off-the-shelf nerve products that overcome the limitations of nerve autografting.

REPORTABLE OUTCOMES: None

REFERENCES: None

APPENDICES: None